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Review

# Mitochondrial calcium as a key regulator of mitochondrial ATP production in mammalian cells

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ABSTRACT

Mitochondrial $Ca^{2+}$ transport was initially considered important only in buffering of cytosolic $Ca^{2+}$ by acting as a "sink" under conditions of $Ca^{2+}$ overload. The main regulator of ATP production was considered to be the relative concentrations of high energy phosphates. However, work by Denton and McCormack in the 1970s and 1980s showed that free intramitochondrial $Ca^{2+}$ ($[Ca^{2+}]_m$) activated dehydrogenase enzymes in mitochondria, leading to increased NADH and hence ATP production. This leads them to propose a scheme, subsequently termed a "parallel activation model" whereby increases in energy demand, such as hormonal stimulation or increased workload in muscle, produced an increase in cytosolic $[Ca^{2+}]$ that was relayed by the mitochondrial $Ca^{2+}$ transporters into the matrix to give an increase in $[Ca^{2+}]_m$. This then stimulated energy production to meet the increased energy demand. With the development of methods for measuring $[Ca^{2+}]_m$ in living cells that proved $[Ca^{2+}]_m$ changed over a dynamic physiological range rather than simply soaking up excess cytosolic $[Ca^{2+}]$, this model has now gained widespread acceptance. However, work by ourselves and others using targeted probes to measure changes in both $[Ca^{2+}]$ and [ATP] in different cell compartments has revealed variations in the interrelationships between these two in different tissues, suggesting that metabolic regulation by $Ca^{2+}$ is finely tuned to the demands and function of the individual organ.



## 1. Introduction

$Ca^{2+}$ has been recognised as a potential regulator of metabolism for 50 years, since Krebs discovered that it activated phosphorylase kinase in 1959 [1–3]. Mitochondria were initially considered as "$Ca^{2+}$-sinks", having the ability to take up large amounts of $Ca^{2+}$, thought to occur only under conditions of cytosolic $Ca^{2+}$-overload (for a historical account see review by Carafoli [4]). Subsequently in the 1960s and 70s, $Ca^{2+}$ at physiological concentrations was found to regulate dehydrogenase enzymes of mitochondria: glycerophosphate dehydrogenase [5], responsible for shuttling reducing equivalents from cytosolic NADH into mitochondria, and the mitochondrial matrix dehydrogenases pyruvate dehydrogenase (PDH) [6], isocitrate dehydrogenase (ICDH)[7] and oxoglutarate (or α-ketoglutarate) dehydrogenase (OGDH) [8]. Thus $Ca^{2+}$ stimulates both glycogen breakdown and glucose oxidation leading to increased ATP supply.

$Ca^{2+}$ uptake and efflux in mitochondria were identified as separate pathways (reviewed in [4]), but they were considered to be important in regulating cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$), and therefore in regulating metabolic processes in the cytoplasm [9]. However, work by Richard Denton and Jim McCormack in the 1970s and 1980s showing that $Ca^{2+}$ activated dehydrogenase enzymes in mitochondria lead them to propose a scheme whereby increases in $[Ca^{2+}]_c$ were relayed to the mitochondria resulting in activation of mitochondrial dehydrogenases and stimulation of ATP synthesis (reviewed in McCormack et al. [10], and see chapter by Denton in this volume). This "parallel activation model" provides a mechanism whereby $Ca^{2+}$ stimulates energy-consuming processes induced by various hormones, muscle contraction, or increased workload in the heart, but also provides the means whereby cells are able to increase ATP supply to keep pace with this increased demand. Previous measurements of total $[Ca^{2+}]$ in mitochondria gave high values, in the mM range. However, later methods (see below) revealed that the free intramitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) is in the nM–μM range, which is in the same range as that for activating dehydrogenases, and similar to free $[Ca^{2+}]_c$. Thus the role of the mitochondrial $Ca^{2+}$ transporters under physiological conditions is to regulate intramitochondrial $[Ca^{2+}]$, and not simply to buffer cytosolic $[Ca^{2+}]$ [11].

This idea was slowly accepted in the field, and the whole area of mitochondrial $Ca^{2+}$ signalling gained a resurgence of interest with the development, in 1992 of a technique for measuring $[Ca^{2+}]_m$ using the photoprotein aequorin targeted to mitochondria [12]. This, together with the prior development of fluorescent indicators [13] and more recently genetically encoded fluorescent probes [14],

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sparked a great deal of work and interest in this area, not only in the role of $[Ca^{2+}]_m$ as a regulator of metabolism, but also its role in controlling whole-cell $Ca^{2+}$-signalling (see other chapters in this volume). These techniques, together with those for measuring $[ATP]_m$ and $[ATP]_c$ using targeted luciferase[15–17], have revealed more about the relationship of $Ca^{2+}$ regulation of mitochondrial ATP production.

In this chapter we will focus specifically on the role of $[Ca^{2+}]_m$ in regulating ATP production by mitochondria under physiological conditions, and the interrelationships of $Ca^{2+}$, NAD(P)H and ATP in different cell types, particularly the heart. What seems apparent is that although similar mechanisms operate in different tissues, spatial organisation of mitochondria and temporal variations in ATP, $[Ca^{2+}]_m$ and redox state of the cells is different in different cell types, suggesting that although the control mechanisms are similar, there are variations in their interrelationships in different tissues. We will not discuss the role of $Ca^{2+}$ and the mitochondrial permeability transition pore (MPTP) in cell death due to either necrosis or apoptosis, referring the reader to other reviews in this issue and elsewhere [18–22].

## 2. Regulation of ATP synthesis in mitochondria

### 2.1. Early work

Oxidative phosphorylation is the process whereby fuel oxidation leads to generation of ATP by the mitochondria: Fuel catabolism produces NADH and $FADH_2$ via mitochondrial dehydrogenases; these transfer electrons ultimately to oxygen via the electron transport chain. The energy of electron transfer is utilised to pump protons out of the mitochondria, creating a pH gradient and membrane potential, together known as the proton-motive force. This drives protons through the ATP synthase back into the mitochondria, resulting in formation of ATP. Transfer of ATP out of mitochondria is via the adenine nucleotide exchanger. Below is the overall reaction, although estimates of the number of ATP molecules synthesised per molecule of NADH vary:

$$NADH + H^+ + O_2 + 2.5\,ADP + 2.5\,P_i \rightarrow NAD^+ + H_2O + 2.5\,ATP.$$

Oxidative phosphorylation in mitochondria provides most of the ATP in nearly all cell types, and accounts for over 90% of ATP production in the heart [23]. It can potentially be regulated by several different mechanisms; from the equation above, increases in $NADH/NAD^+$ and ADP/ATP would be expected to increase drive the forward reaction. Oxygen is not rate limiting until levels below about 20 μM [10], a level much lower than that occurring physiologically. Thus it was originally proposed that changes in the ADP/ATP ratio were the main regulator of ATP synthesis [24], and this was easily demonstrated in isolated mitochondria. However, the role of a drop in ATP and increase in ADP as the trigger for stimulating ATP synthesis *in vivo* had to be re-evaluated when studies started demonstrating that high energy phosphate levels did not change under conditions that imposed a large energy demand on a tissue, and hence would be expected to produce a drop in ATP. For example in studies on whole beating hearts using NMR found ATP, $P_i$, phosphocreatine levels to remain remarkably constant, even under conditions where oxygen consumption increased approximately 3-fold [25,26]. ADP could not be measured directly, but calculated levels using the creatine kinase equilibrium indicated that it did not change either [26]. The free [ADP] in beating hearts was calculated to be around 50 μM [26], which represents an average value from the whole heart. To achieve the observed increase in respiration, of 2.5-fold, when the hearts were stimulated with adrenaline, Katz et al. [26] estimated that [ADP] would have to increase by about 4-fold, and this was not observed in any of their experiments. This suggested that other factor(s) had to be involved.

### 2.2. Activation of dehydrogenases by $Ca^{2+}$

Of course there had to be an increase in ATP production when cells and tissues were stimulated, but it seemed that the process was very finely tuned, so that ATP supply was almost exactly matched to ATP utilisation. Work by Denton and others on $Ca^{2+}$ regulation of mitochondrial dehydrogenases leads them to propose that an increase in $[Ca^{2+}]_m$, resulting from a rise in $[Ca^{2+}]_c$ that occurred upon increased workload or agonist stimulation, increases NADH supply and hence ATP synthesis; thus providing the link between energy supply and demand (see review by Denton in this issue).

Although this elegant theory is now widely accepted, it met with resistance at the time, partly due to lack of direct methods for measuring $[Ca^{2+}]_m$ *in vivo* and of demonstrating that this was in a range capable of stimulating dehydrogenase activity. However, McCormack and colleagues had found that activation of PDH occurred in whole hearts upon inotropic stimulation [27,28], and that this was prevented by ruthenium red (RuR) [29,30], although there are problems with using this (see below): "These results provide further evidence that the activation of PDH by positive inotropes is accomplished by, and at least in part due to, raised mitochondrial matrix free $[Ca^{2+}]$ and that such increases can be maintained in isolated and suitably incubated mitochondria" [31].

### 2.3. ATP synthase

Several studies have suggested that dual control by $Ca^{2+}$ may exist at the level of the dehydrogenases (described above) and the ATP synthase [23,32,33]. Attempts to correlate the rate of ATP synthesis with the levels of phosphates, NADH or $\Delta\mu H^+$ in the heart have failed (reviewed by Balaban [34]), indicating that the ATP synthase cannot simply be responding to changes in levels of its substrates. For example; electrical stimulation of adult rat cardiomyocytes leads to approximately doubling of ATP synthase capacity in 1–2 min, but this was abolished in the presence of RuR [32]. Territo et al. [33] suggested a direct activation of the ATPase by $Ca^{2+}$ with a Km of 200 nM, well within the physiological range. But as yet there is no mechanism as to how $Ca^{2+}$ activates the ATPase, although involvement of specific $Ca^{2+}$-binding proteins that then regulate the ATPase has been proposed [35,36].

### 2.4. Mitochondrial $Ca^{2+}$ transport pathways and overview of regulation

Work on isolated mitochondria suggested that the mitochondrial $Ca^{2+}$-uniporter (CaUP), was a relatively slow $Ca^{2+}$-uptake pathway because of its low affinity for $Ca^{2+}$, and the efflux pathway, the $Na^+/Ca^{2+}$-exchanger (mNCX), even slower [37,38] (see review by Sheu and Gunter in this issue). Later work in the 1990s revealed that the slow transport by the CaUP could be overcome by having mitochondria situated near $Ca^{2+}$ release channels of the endoplasmic reticulum (ER), where they would be exposed to microdomains of high $Ca^{2+}$ allowing rapid mitochondrial uptake (reviewed by Rizzuto in this volume). Fig. 1 gives an overview of the role of $[Ca^{2+}]_m$ in regulating ATP supply and demand.

## 3. Relationship between mitochondrial $[Ca^{2+}]$, NADP(P)H and [ATP] in living cells and tissues

### 3.1. Heart

#### 3.1.1. Mitochondrial $Ca^{2+}$ measurements in the heart

A question that remained unanswered for some time, and is still controversial, is whether or not $[Ca^{2+}]_m$ changes on a beat-to-beat

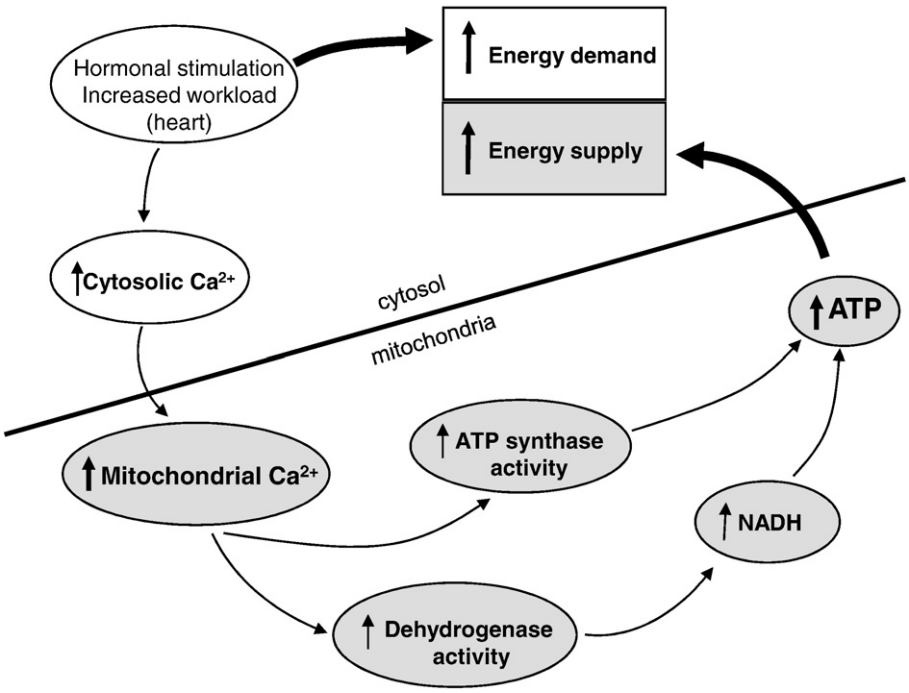


**Fig. 1.** Mitochondrial $Ca^{2+}$ — key role in regulation of energy supply and demand.

basis in the heart. Estimates of myocardial free $[Ca^{2+}]_m$ vary from about 50–150 nM at rest (during diastole) to about 500–2000 nM at the peak of contraction, or upon stimulation with an agonists or increased workload. Based on work in isolated mitochondria, the CaUP and mNCX pathways could certainly not respond quickly enough to the very rapid (millisecond) changes in $[Ca^{2+}]_c$ that occur during excitation-contraction (EC) coupling. However, more recent work using isolated myocytes has suggested that mitochondrial $Ca^{2+}$ transients do occur during EC coupling in neonatal and adult cardiac myocytes [39–41], although there is conflicting data on this in the literature since other studies reported that mitochondrial $Ca^{2+}$ accumulation occurred over tens of seconds in cardiac myocytes [42–44]. Part of the conflict may be due to different species or stage of development (neonate versus adult), however these equally represent an explanation for the differences although the implications for having mitochondrial $Ca^{2+}$ spiking in some species but not others then present a different question.

Another hurdle has been the lack of specific inhibitors of the mitochondrial $Ca^{2+}$ transporters that can be used in cardiomyocytes. RuR has non-mitochondrial effects, notably on the sarcoplasmic reticulum (SR); in isolated myocytes it initially inhibits cell contraction, but at higher concentrations (above about 10 μM) causes cytosolic $Ca^{2+}$ waves and eventual cell hypercontracture [45], although at these concentrations it does inhibit $Ca^{2+}$ entry into mitochondria [42,46]. However, in whole heart studies protective effects of RuR against myocardial ischaemia/reperfusion injury have been reported [47,48], and McCormack and England found that 2.5 μg/ml ruthenium red (which would correspond to about 1–2 μM) could block increases in mitochondrial pyruvate dehydrogenase activity without altering activation of phosphorylase (a cytosolic enzyme) upon stimulation of hearts with adrenergic agonists [29]. Allen et al. [49] pre-perfused isolated rat hearts with fura-2/AM before induction of hypoxia and reoxygenation and found 2.5 μM ruthenium red could inhibit the reoxygenation induced rise in $[Ca^{2+}]_m$; however, no functional effects were reported and it is possible that this was an indirect result of a decrease in $[Ca^{2+}]_c$. Why RuR should be more effective in isolated hearts as opposed to isolated myocytes remains unclear.

Ru360 is more specific for mitochondria than RuR, but we and others have found that it does not appear to enter either neonatal [40,41] or adult [41] myocytes. Other groups have, however, reported success in using Ru360 (see Section 5 below). We have used clonazepam in adult cardiomyocytes as a relatively specific inhibitor of the mNCX [50], although in adult guinea-pig myocytes and neonatal rat heart cells it inhibited contraction (CJ Bell and EJ Griffiths, unpublished data), and so we could not use it in these cells to probe mitochondrial function.

### *3.1.2. NAD(P)H as an indicator of ATP production*

Due to the difficulty of measuring [ATP] in living cells, NAD(P)H levels have been used as an indirect indicator of energetic state in whole hearts [26,51], myocytes [52] or trabeculae [53]. This is done using cell autofluorescence, so cannot distinguish between NADH and NADPH, but it has been shown that over 80% of the signal originates from mitochondria in the heart [54]. It is also important in these studies to ensure that oxygen supply is not rate limiting, as that would result in increased NAD(P)H due to inhibition of the respiratory chain [34]. In well-oxygenated hearts, no change in NAD(P)H was observed in response to physiological increases in workload [51]. Additionally, altering the substrate from glucose to pyruvate increased NAD(P)H, and this was associated with an increase in the $ATP/ADP+P_i$, and increases in $O_2$ consumption [55]. However, although lactate gave the same increase in NAD(P)H, there was no rise in $ATP/ADP+P_i$. So similar levels of NAD(P)H were associated with different $ATP/ADP+P_i$, and so an increase in NAD(P)H cannot be taken as an unambiguous indicator of increased ATP production [55].

In myocytes, conflicting results have been obtained as to whether NAD(P)H changes upon rapid stimulation of cells: increases, no change and decreases have all been reported [43,52,56]. In isolated trabeculae, NAD(P)H was more carefully measured by using an internal reference that negated motion artefacts. When the muscle strips were stimulated at 3 Hz from rest, an initial drop in NAD(P)H occurred within 5 s, followed by an increase to the initial or higher levels over 60 s [57]. However, under physiological conditions, the heart is never likely to be subjected to such a sudden and steep increase in energy demand (i.e. from rest to beating rapidly). Thus

NAD(P)H measurements in the heart cannot be used as an indicator of an increase in ATP synthesis.

*3.1.3. ATP measurements*

In rat hearts, about 2% of cell ATP is consumed with each heart beat, and under physiological conditions in well-oxygenated hearts, over 90% of its ATP is made by oxidative phosphorylation [23]. Changes in work rate (ATP utilisation) may be 5–10 fold in vivo, but ATP levels remain remarkably constant as discussed [25,26] above. Changes in ATP are initially buffered by the phosphocreatine system:

$$\text{ADP} + \text{phosphocreatine} \xrightarrow{\text{creatine kinase}} \text{ATP} + \text{creatine}.$$

But this only lasts for a few seconds, so there must be a mechanism for increasing ATP synthesis rapidly enough to respond to ATP consumption. Another problem in elucidating the relationship between $[Ca^{2+}]_m$ and [ATP] was that although ATP has been measured in whole hearts, and now in animals and humans using non-invasive $^{31}$P-NMR (reviewed in [34]), only relatively slow responses were measured, and so it could not be determined whether ATP was varying beat-to-beat, or during the time taken for mitochondria to accumulate $Ca^{2+}$ to a level sufficient to activate the dehydrogenases. In one study luciferase was microinjected into single cardiomyocytes and rapid depletion of (cytosolic) ATP levels in response to an uncoupler were observed [58], but changes in response to physiological conditions, or in response to normal cell contraction, were not reported. Furthermore, free ATP levels have not been directly measured in different compartments of the living cardiomyocyte, and the relationship between mitochondrial [ATP] ($[ATP_m]$) and cytosolic ATP ($[ATP]_c$) is not known.

*3.1.4. Measurement of mitochondrial and cytosolic [$Ca^{2+}$] and [ATP] in cardiac myocytes using targeted probes*

In a recent paper we expressed aequorin and luciferase in either mitochondrial or cytosol in adult cardiomyocytes, the first time this has been done [41]. Although it was not possible to measure [ATP] and $[Ca^{2+}]$ simultaneously, we carried out parallel experiments on small populations of cells under identical conditions where myocytes were electrically stimulated to contract synchronously. As well as the expected beat-to-beat changes in $[Ca^{2+}]_c$, we also observed beat-to-beat changes in $[Ca^{2+}]_m$, the amplitude of which was increased in the presence of an adrenergic agonist. However, these transients were only discernible at elevated external $[Ca^{2+}]$; at lower, physiological (1–2 mM) external $[Ca^{2+}]$ it was difficult to detect transients, possible due to the low expression of aequorin in mitochondria compared with that in the cytosol. Nevertheless, both higher external $[Ca^{2+}]$, and/or presence of an adrenergic agonists showed clear mitochondrial $Ca^{2+}$ transients (Fig. 2). In neonatal cells, where mAq was well-expressed, we observed clear mitochondrial $Ca^{2+}$ transients even under basal conditions [41], agreeing with a previous report [40].

We observed no beat-to-beat changes (i.e. >1%) in free [ATP] in either cytosol or mitochondria using luciferase targeted to these compartments in adult myocytes [41]. Neither could we observe changes upon addition of isoproterenol to cells that were already beating, suggesting that ATP supply is well matched to the increased demand caused by the isoproterenol-induced increase in contractile force. Thus $[ATP]_c$ was extremely well buffered in myocytes, even under conditions where the cells were stimulated to beat rapidly in presence of an adrenergic agonist to further increase ATP demand by the cell [41]. This is in agreement of studies using whole hearts described above [51,55].

However, when we allowed cells to rest, then suddenly stimulated to contract in the presence of isoproterenol, $[ATP]_m$ showed a significant transient drop (up to 22% in some cells) followed by recovery to higher than initial levels [41]. These changes seem likely to

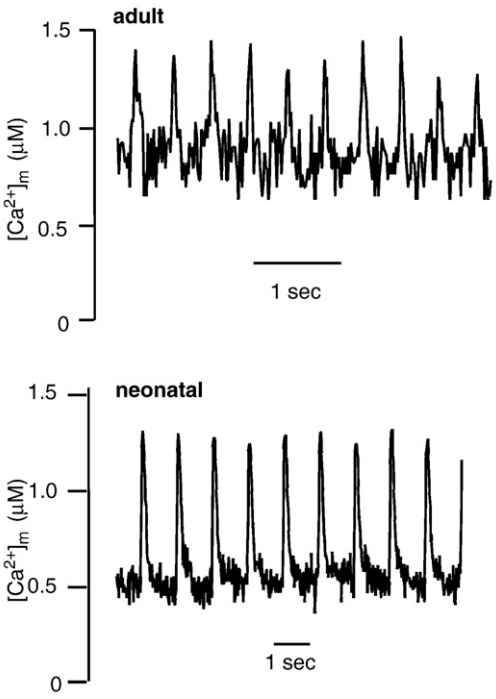


**Fig. 2.** Beat-to-beat spikes of mitochondrial $Ca^{2+}$ in adult and neonatal cardiomyocytes. $[Ca^{2+}]_m$ was measured using targeted aequorin and calibrated as described in [41]. Both cell types were exposed to 4 mM external $[Ca^{2+}]$ and isoproterenol. Adult myocytes were stimulated to contract at 2 Hz; neonatal cells beat spontaneously at about 1 Hz. Data taken from [41].

reflect an initial activation of ATP-requiring processes in the cytosol, such as ion pumps and contractile proteins, which would causes a drop in $[ATP]_m$ before a time-dependent activation of mitochondrial oxidative metabolism by $Ca^{2+}$ [16,59] stimulated ATP synthesis. The changes in $[ATP]_c$ were much smaller than those in the mitochondrial matrix, again suggesting that even when $[ATP]_m$ does change, the cytosolic energy supply is very rapidly matched to the increased demand.

Since ATP levels are falling, and presumably ADP rising, during the initial few seconds when cells are stimulated from rest, then ADP could provide the stimulation of ATP synthesis required to keep $[ATP]_c$ relatively constant during this period. Also, the average $[Ca^{2+}]_m$ takes 30–60 s to reach its maximum value, and so again would not stimulate dehydrogenases and NADH production during the initial phase. Only after this occurred would $[ATP]_m$ increase again. The time courses for *average* $[Ca^{2+}]_m$ and $[ATP]_m$ have been overlaid in Fig. 3 to illustrate these points.

An important caveat must, however, be made that since we compared changes in ATP (luciferase) and $Ca^{2+}$ (aequorin), after one or two days of culture, respectively, there may be subtle changes in cell physiology between these two time points which might affect the changes in these two parameters following cell stimulation.

This work indicated, for the first time that ATP levels in cytosol and mitochondria respond differently to changes in ATP demand in these compartments. A possible mechanism for this effect was suggested by our observation that cardiomyocytes exhibited rapid $Ca^{2+}$ pulses during the contractile cycle. These rapid $Ca^{2+}$ transients are apparently decoded by the matrix dehydrogenases into a time-averaged signal that stimulates ATP production to keep pace with ATP demand, in a manner analogous to the decoding of mitochondrial $Ca^{2+}$ transients in liver [60] and other cell types [61]. Thus, stimulated mitochondria maintain a constant phosphorylation potential in the cytosol. This fits with the "parallel activation model" [34] whereby $Ca^{2+}$ is the key link — simultaneously stimulating ATP-consuming processes in the SR, sarcolemma and

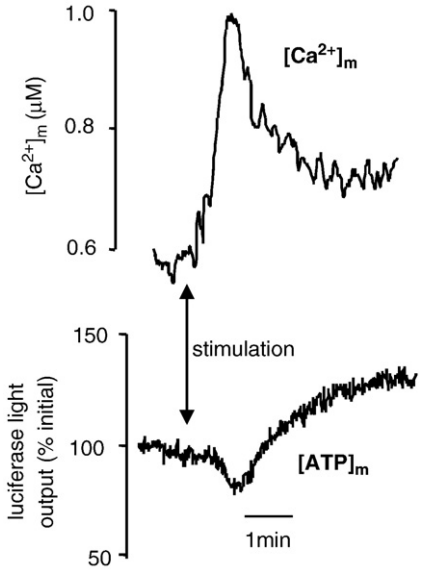


**Fig. 3.** Changes in $[ATP]_m$ and average $[Ca^{2+}]_m$ in adult cardiomyocytes stimulated to beat form rest. $[Ca^{2+}]_m$ and $[ATP]_m$ were measured using targeted aequorin and luciferase, respectively, in parallel experiments on small populations of cells stimulated to contract at 2 Hz from rest. In this figure the average $[Ca^{2+}]_m$ is shown, and so mitochondrial $Ca^{2+}$ transients are not visible as they are in Fig. 2. Figure modified form data first published in [41].

cytosol with ATP production in mitochondria, first proposed by McCormack and Denton [27]. Classical mediators, like ATP, ADP and $P_i$ would also play a role, as seen in the experiments here where ATP demand is abruptly increased, and $[ATP]_m$ falls, before $[Ca^{2+}]_m$ rises to levels sufficient to activate dehydrogenases.

### *3.1.5. Importance of subcellular organisation in mitochondrial $Ca^{2+}$ transport and energy regulation*

Studies from many other cell types have indicated that the subcellular organisation of mitochondria is vital in determining their $Ca^{2+}$ handling properties [12,62]. Indeed, Sharma et al. [63] found that in skinned myocytes application of caffeine to the cells caused a large release of $Ca^{2+}$ from the SR that was taken up by mitochondria, as well as entering the cytosol, and the mitochondrial uptake was inhibited by RuR. Thus it is conceivable that mitochondria located in close proximity to $Ca^{2+}$ channels on the SR or plasma membrane are exposed to a much higher level of $[Ca^{2+}]$ than those elsewhere in the cytosol [64], accounting for the ability of at least a subpopulation of mitochondria to take up $Ca^{2+}$ on a beat-to-beat basis as we observed using targeted aequorin [41]. Full discussion of the role of the mitochondria $Ca^{2+}$ transporters in modulating whole cell $Ca^{2+}$ signalling is covered in the article by Sheu and Gunter in this issue, and recent reviews [65,66].

In addition to the known proximity of mitochondria to the ER in a variety of cell types, there is now evidence for a direct physical coupling that persists during isolation of mitochondria (reviewed in [67]). This evidence was provided in the heart by isolation of mitochondrial fractions with associated SR components that were highly resistant to purification [64]. These "SR appendices" were capable of transferring $Ca^{2+}$ directly to mitochondria (measured with rhod-2 fluorescence) upon stimulation with caffeine, and of producing an increasing in the NADH autofluorescence signal, suggesting activation of the dehydrogenases. This is the first direct evidence that $Ca^{2+}$ transfer directly from the SR to mitochondria is capable of activating oxidative metabolism [64].

As well as being important in mitochondrial $Ca^{2+}$ dynamics, there is evidence that the subcellular arrangement of mitochondria with other cell compartments or enzymes, notably the ATPases, can also play a role in regulating energy metabolism by ADP and ATP. That the concentration of adenine nucleotide phosphates may differ in regions around mitochondria was revealed by Saks et al. [68] found that the apparent Km for exogenous ADP stimulation of respiration at low $[Ca^{2+}]$ (0–1 μM) was higher in permeabilised cardiomyocytes, 250–350 μM, compared with that in isolated mitochondria, approximately 20 μM. This and subsequent work led them to propose that mitochondria are closely associated with structures such as SR and sarcolemma containing ATPases and are organised into "intracellular energy units" (ICEUs) [69–71], and they found that cytoskeletal proteins also played a role in maintaining the structure of the ICEUs [72]. However, in permeabilised cardiomyocytes exposed to $[Ca^{2+}]$ of about 1 μM, where the cell underwent contracture and where mitochondrial $[Ca^{2+}]_m$ also rose, there was a marked decrease in the Km of exogenous ADP for respiration [73], similar to that observed in isolated mitochondria. The authors concluded from this that contracture modified the intracellular structure of the mitochondrial association with other organelles sufficiently to remove the diffusion limits of ADP seen in the resting cells [73]. Similar evidence for the existence of ICEUs had been found in skeletal (red) muscle [74]. However, in a non-beating cultured cardiac cell line, where mitochondria are not organised as in adult cardiomyocytes and instead form a more tubular network seen in many types of non-muscle cell, the Km of ADP for respiration was similar to that in isolated mitochondria, indicating absence of ICEUs in these cells[75].

This group also argues that $[Ca^{2+}]_m$ may not be the sole factor, or possibly not a major factor, in regulating ATP supply and demand in the heart, since modelling studies have predicted that the increase in $[Ca^{2+}]_m$ in the heart would only be enough to stimulate respiration 2–3 fold, whereas increases of 10–20 fold are seen in vivo [33,76,77]. Therefore localised regulation of [ADP] in the ICEUs would present a method of stimulating ATP synthesis by mitochondria without changes in bulk [ADP] or [ATP] [73]. However, extrapolating these studies to the physiological situation of rapidly beating cardiomyocytes, either cells or whole hearts, that are continually shortening and relengthening is going to be difficult experimentally. If the authors are correct, the theory also implies that control of respiration by ADP is operating on a millisecond timescale (in rat cardiomyocytes, for example). Alternatively respiration could be sensitive to the time-averaged local [ADP], in a similar manner as we suggested above for rapid changes in $[Ca^{2+}]_m$. Nevertheless, some evidence for such gradients of ADP has been provided using NMR combining different protocols of magnetization transfer and biochemical measurements [78]; calculated gradients of ADP of 20–100 μM occurred in the compartment between mitochondria, cytosol and myofibrillar ATPases [78].

## *3.2. Other cell types*

### *3.2.1. Overview*

In other cell types, regulation of ATP production by $[Ca^{2+}]_m$ may follow a different timecourse, and contribute to a different extent. In the heart, ATP levels are initially buffered by the phosphocreatine system, but in other cells such as liver, this is not present and so a fall in ATP levels may play an important role at least in the early phase of cell stimulation, although $Ca^{2+}$ activation of dehydrogenases still seems to be the dominant factor in maintaining the ATP supply.

### *3.2.2. HeLa cells*

A key paper by Jouaville et al. [79] used aequorin and luciferase targeted to mitochondria to demonstrate a direct link between $[Ca^{2+}]_m$ and $[ATP]_m$: When HeLa cells were stimulated with histamine, which releases $Ca^{2+}$ from intracellular stores via $IP_3$, there were large increases in both $[Ca^{2+}]_m$ and $[ATP]_m$. Histamine also caused a transient drop, followed by sustained increase, of $\Delta\psi_m$. Preventing the rise in $[Ca^{2+}]_m$ (using BAPTA) also prevented rise in $[ATP]_m$, this proving a direct link between

the two. They also provided evidence for a mitochondrial "memory" mechanism: following stimulation with histamine, $[Ca^{2+}]_m$ rose and then fell again upon histamine washout. Challenging the cells with pyruvate and lactate following the washout produced an increase in $[ATP]_m$ similar to that seen in presence of histamine, even though $[Ca^{2+}]_m$ had now returned to basal levels. This was maintained for 30 min after the initial histamine exposure. The mechanism of this "memory" is unknown, but possibly involves increases in mitochondrial volume [80], prolonged changes in $\Delta\psi_m$ or activation of ATP synthase by $Ca^{2+}$-binding regulatory proteins (see above). So there was a strict dependence of $[ATP]_m$ on $[Ca^{2+}]_m$, which can therefore relay changes in cell energy consumption to the mitochondria.

Since HeLa cells are a cancer cell line and highly glycolytic, the authors also performed some experiments in skeletal muscle myotubes, and reached similar conclusions, i.e. that and increase in $[Ca^{2+}]_c$, this time induced by depolarisation of the myotubes, could trigger a rise in $[ATP]_m$ [79]. A similar phenomenon was observed in a pancreatic β-cell line that is almost entirely aerobic [81].

*3.2.3. Pancreatic islet cells and hypothalamic neurons*

Pancreatic islet cells, notably insulin-secreting β and glucagon-secreting α cells, represent an interesting alternative physiological paradigm to cardiac and other cells where the role of ATP synthesis is "purely" to drive ATP consuming process. In these fuel-sensing neuroendocrine cells, ATP synthesis also plays a central role in signal transduction by nutrients including glucose. Thus, enhanced mitochondrial oxidative activity is required not only to fuel hormone synthesis, secretory granule trafficking and finally $Ca^{2+}$-stimulated exocytotic release of insulin [82–84], but is also deployed as part of the machinery by which changes in the plasma concentrations of glucose and other secretagogues are detected [85,86]. Thus in pancreatic β cells, the role of $Ca^{2+}$ in regulating mitochondrial metabolism is particularly complex and still somewhat controversial [87]. Here, enhanced glycolytic flux and stimulation of citrate cycle activity increase cytoplasmic ATP/ADP ratio, leading to closure of ATP-sensitive $K^+$ ($K_{ATP}$) channels on the plasma membrane [88], inhibition of AMP-activated protein kinase [89], plasma membrane depolarisation, and the influx of $Ca^{2+}$ ions through voltage-sensitive L-type $Ca^{2+}$ channels (Cav1.2 and Cav1.3) that then triggers insulin secretion (reviewed by Rutter [85]). Note that $Ca^{2+}$-induced $Ca^{2+}$ release from internal endoplasmic reticulum [90] – or more probably secretory granule [91] – associated stores may also be involved. This, in turn, leads to increases in free intramitochondrial $Ca^{2+}$ concentration, as first reported using a mitochondrially-targeted aequorin [92,93] and more recently with a mitochondrially-targeted GFP-derived "pericam" (Ravier, M.A. and Rutter, manuscript under revision), and an apparent increase in mitochondrial and cytosolic free ATP concentrations as reported with recombinant luciferases and photon-counting imaging [16,59,94].

Interestingly, both the cytosolic $Ca^{2+}$ increase [93,95,96] and the changes in cytosolic and mitochondrial free ATP concentration in response to elevated glucose concentrations [94] are oscillatory, matching "slow wave" oscillations in insulin secretion (2–3 min periodicity). These observations have led to two alternative models in which either (a) elevated mitochondrial $Ca^{2+}$ concentrations, through activating citrate cycle enzymes, the respiratory chain and ATP synthase [10], leads to elevated ATP delivery to the cytoplasm, further reinforcing the closure of $K_{ATP}$ channels in the plasma membrane and amplifying $Ca^{2+}$ influx or (b) that elevated cytosolic $Ca^{2+}$ drives enhanced ATP consumption in this compartment (for $Ca^{2+}$ extrusion, granule movement etc.) and may lead to a decrease in mitochondrial proton motive force due to uptake through the uniporter[97]. Parallel oscillations of intracellular $Ca^{2+}$ activity and mitochondrial membrane potential have been observed in mouse pancreatic β-cells [98]. Arguing for possibility (a), recent studies [99] in which mitochondrial $Ca^{2+}$ changes were attenuated by over-expression in the mitochondrial matrix of the $Ca^{2+}$ binding protein S100G, diminished glucose-induced increases in mitochondrial membrane and cytoplasmic ATP concentrations. Hence, in the β cell system, $Ca^{2+}$ uptake into mitochondria appears to form part of a feed-forward loop, which enables mitochondrial ATP synthesis not only to be enhanced to meet the increased demands for cellular work, but to further amplify a nutrient-activated signalling mechanism to ensure normal stimulus-secretion coupling. Whether activation of mitochondrial fluxes, for example through the inhibition of the mNCX with CGP-37157 [100] or by stimulation of PDH activity [38], represent potential pharmacological targets for the treatment of deficient insulin secretion in type 2 diabetes, is an interesting possibility.

In pancreatic α cells, elevated glucose concentration leads to increases in mitochondrial and cytosolic ATP levels though in these cells coordinated regulation of glucagon release appears to involve both a direct action and a metabolic action of the sugar [101]. This possibly involves more minor changes in $K_{ATP}$ channel activity [102], but also signalling from neighbouring β cells via secreted products such as $Zn^{2+}$ ions [103] and γ-amino butyric acid (GABA) [104–106].

Finally, glucose-sensitive neurons in the mediobasal hypothalamus associated with the control of feeding, satiety and responses to hypoglycaemia appear also to engage changes in mitochondrial ATP synthesis as part of a fuel-sensing system [94,106]. The inter-relationships between $Ca^{2+}$ influx, ATP synthesis neuronal firing and the release of neuropeptides remain to be examined in detail in this system.

*3.2.4 Liver*

Liver is a highly metabolically active tissue, with processes such as gluconeogenesis being regulated by both substrate/product levels as well as by hormones such as glucagon and vasopressin, the latter stimulating $Ca^{2+}$ release from intracellular stores. Studies using isolated hepatocytes have revealed a complex relationship between $[Ca^{2+}]_c$, $[Ca^{2+}]_m$ and NAD(P)H levels, depending on the amplitude and duration of the initial change in $[Ca^{2+}]_c$: Hajnoczky et al. [60] measured $[Ca^{2+}]_c$ (using fura-2), NADP(H), and flavoprotein (FAD) fluorescence simultaneously in single primary hepatocytes. FAD is predominantly mitochondrial, and since there is an intrinsic FAD-containing subunit of OGDH and PDH, they used FAD fluorescence as a direct indicator of dehydrogenase activity.

Upon stimulation with vasopressin, they observed a spike of $[Ca^{2+}]_c$, lasting approximately 50 s. The up-phase of the spike triggered a slower increase in NAD(P)H and decrease of FAD, which took much longer to return to pre-stimulation values, about 300 s. These $[Ca^{2+}]_c$ changes were transmitted into the mitochondria, which also underwent $Ca^{2+}$ spiking (measured with rhod-2), over a similar, slightly prolonged, timescale to the cytosolic spikes. However, with repetitive cytosolic $Ca^{2+}$ spiking, although mitochondrial spikes occur, the NAD(P)H fluorescence is maintained at an elevated level. Thus indicating that "frequency-modulated $[Ca^{2+}]_m$ signals are translated into a time-averaged redox response…" [60]. Interestingly, with a sustained, non-oscillatory, rise of $[Ca^{2+}]_c$, the mitochondrial $[Ca^{2+}]$ declines after the initial rise. They proposed that this was due to lack of release of $Ca^{2+}$ from ER stores into mitochondria. However, the NAD(P)H declined more slowly than $[Ca^{2+}]_m$, suggesting that either there were other factors affecting mitochondrial metabolism, or that the activity of the dehydrogenases declines more slowly than $[Ca^{2+}]_m$.

In a subsequent paper, this group assayed PDH synchronously with NAD(P)H following stimulation of hepatocytes with vasopressin [107]. They observed similar results as before, namely that a prolonged increase in $[Ca^{2+}]_c$ gave rise to a transient increase in $[Ca^{2+}]_m$, the NAD(P)H signal declining more slowly than $[Ca^{2+}]_m$. PDH activity increased rapidly, before plateauing briefly followed by a second sustained increase that persisted even when $[Ca^{2+}]_m$ and NAD(P)H had returned to baseline values [107]. Vasopressin caused a rapid and sustained decrease in [ATP] by about 15%. They suggested that this

decrease in ATP may act together with $[Ca^{2+}]_m$ to give the first phase of PDH activation. However, they reckoned that this would not cause the second increase in PDH activity, since ATP levels plateaued, rather the fall in NAD(P)H would tend to inhibit PDH kinase, thus activating PDH. Further experiments showed that the second phase of PDH activation by vasopressin could be explained by combined effects of an increase in cytosolic ATP consumption, leading to a fall in $[ATP]_m$, and subsequent reoxidation of NAD(P)H, mediated by an increase in respiratory chain activity. Vasopressin also induced an increase in $\Delta\psi_m$ in the hepatocytes; this rise was more prolonged than changes in NAD(P)H, reaching a peak value about 600 s.

Another mechanism operating in liver is that whereby hormones that increase $[Ca^{2+}]_c$ also cause an increase in mitochondrial matrix volume, which significantly affects respiration: Halestrap proposed that the increase in volume involved a $Ca^{2+}$-mediated increase in pyrophosphate (PPi) in the mitochondrial matrix; PPi binding to the adenine nucleotide translocase increases the permeability of $K^+$ through this channel, and causes mitochondria to swell (for full details see review by Halestrap [80]). This mechanism has been demonstrated in response to vasopressin in hepatocytes [108] and perfused liver [109], as well as in isolated mitochondria. Thus it could play a complementary role to dehydrogenase activation by $Ca^{2+}$ in liver. However, in the heart, although increases in volume of isolated mitochondria can stimulate respiration [110], this does not appear to happen *in vivo* as changes in mitochondrial volume could not be detected following perfusion of whole hearts with agonists that increase $[Ca^{2+}]_c$ [111].

## 4. Dysregulation in disease

For diseases of the liver and central nervous system please see other reviews in this issue.

### 4.1. Heart

#### 4.1.1. Ischaemia/reperfusion injury

$[Ca^{2+}]_m$ has long been known to play a role in ischaemia/reperfusion injury in the heart, especially in triggering the permeability transition pore; this is discussed by Halestrap in this volume and we will not consider it further here.

#### 4.1.2. Cardiomyopathies

Hearts from cardiomyopathic hamsters develop heart failure, and exhibit reduced developed pressure and adenine nucleotide content. Perfusion with either pyruvate or isoproterenol restores both these parameters [112]. In myocytes isolated from these hearts, there is a reduction in PDH activity, and also a reduced $[Ca^{2+}]_m$ in response to rapid electrical stimulation compared with control hearts [113]. This seemed likely due to a reduction in systolic $Ca^{2+}$ transient, leading to reduced $[Ca^{2+}]_m$ and failure to activate PDH. Isolated mitochondria also showed slightly reduced $Ca^{2+}$ uptake (although only at higher external $Ca^{2+}$), indicating that a reduced $[Ca^{2+}]_c$ may not be the only mechanism — but these workers concluded that this was unlikely to be physiologically relevant.

However, as discussed above, in other cell types mitochondria are exposed to local high $[Ca^{2+}]$ domains near ER/SR release sites, and this is likely to also be the case in cardiac myocytes [63]; a recent paper therefore re-investigated mitochondrial $Ca^{2+}$ transport in mitochondria from the cardiomyopathic hamsters, with the assumption that a reduction in transport at higher external $[Ca^{2+}]$ would be physiologically relevant, and found that transport was reduced over a range of $[Ca^{2+}]$ from 1–10 μM [114]. This was associated with a reduced $\Delta\psi_m$ and reduced activities of complexes I and IV. It is possible but untested that inhibiting the mNCX in these hearts would be beneficial in restoring $[Ca^{2+}]_m$ and activating PDH.

### 4.2. Complex I deficiency

An intriguing study looked at human mitochondrial complex I deficiency — this is associated with numerous clinical symptoms, ranging from lethal encephalopathy's, of which Leigh disease is the most common, to neurodegenerative disorders, including Parkinson's disease [115]. This study used skin fibroblasts from children with Complex I deficiency, and measured cytosolic $Ca^{2+}$ transients in response to bradykinin [116]. They found impaired increases in $[Ca^{2+}]_c$, $[Ca^{2+}]_m$, and ATP production in response to bradykinin in the complex I deficient fibroblasts. Treatment with 1 μM CGP37157, an inhibitor of mNCX that can be used in some cell types, restored all these parameters.

They suggested that (i) decreased ATP production in this disease is a result of reduced mitochondrial $Ca^{2+}$ uptake, possibly due to a reduced $\Delta\psi_m$ in these patients, and (ii) CGP37157 can indirectly promote enhanced release of $Ca^{2+}$ from the ER in these cells by enhancing mitochondrial $Ca^{2+}$ uptake so that there is less inhibitory $Ca^{2+}$ available at mouth of the $IP_3$-operated ER $Ca^{2+}$ release channels.

Complex I (NADH:ubiquinone oxidoreductase) deficiency is associated with a range of progressive neurological disorders (early onset and often devastating course). This group also found, using fibroblast lines derived from these patients, that $[ATP]_m$, mitochondrial $Ca^{2+}$ handling and ATP production are impaired to a variable extent among these patients[117]. They concluded that reduction in complex I activity leads to depolarisation of $\Delta\psi_m$, decrease in ATP supply to ATPases of intracellular stores (ER), and so to a reduced $Ca^{2+}$ content of these stores. So less $Ca^{2+}$ released in response to a stimulus, so less $[Ca^{2+}]_m$ accumulated and less ATP produced in response to that stimulus.

## 5. Problems remaining

One question that remains unanswered due to lack of specific inhibitors of the mitochondrial $Ca^{2+}$ transporters is whether blocking $Ca^{2+}$ entry into mitochondria makes the heart unable to cope with increased workload. One study addressed this using Ru360 in isolated perfused rat hearts, and found it had no effect on contractile function at <5 μM; above this concentration, contractile force generation was depressed and resting tension elevated [118] — whether this was attributed to mitochondrial or non-mitochondrial effects cannot be determined from this study.

Thus it is difficult to know whether inhibiting mitochondrial $Ca^{2+}$ uptake in a normal beating heart has any detrimental effect, although we can speculate that partial inhibition of mitochondrial $Ca^{2+}$ uptake achieved by low concentrations of Ru360 has no adverse effect, whereas total inhibition, which would occur when using concentrations >5 μM, depresses cardiac function. Even a partial inhibition of CaUP may have a deleterious effect if the heart was stimulated with adrenaline and/or subjected to increased workload, assuming that an increase in $[Ca^{2+}]_m$ under these conditions was essential for increasing ATP supply. However, to the best of our knowledge, this type of experiment has yet to be attempted.

Similarly although the studies above using CGP37157 are in theory promising, this drug cannot be used in all cell types. We also do not know what effect inhibition of mitochondrial $Ca^{2+}$ efflux via the mNCX or uptake via the CaUP would have on whole cell $Ca^{2+}$ homeostasis.

## 6. Future directions

In theory it is now possible to measure $[Ca^{2+}]_m$ in beating hearts using genetically encoded probes, although no-one has yet done this. Another leap forward would be provided by cloning of the mitochondrial $Ca^{2+}$ transporters, as well as development of more specific inhibitors of the $Ca^{2+}$ transport pathways. Although we have

emphasised the importance of $Ca^{2+}$ in regulating mitochondrial ATP production, it is clear that other factors play a role under certain conditions, notably the ATP/ADP ratio, and that the relative importance will vary between tissues. Another current limitation is that free [ADP] cannot be measured, and thus we do not really know how free $[ADP]_c$ and $[ADP]_m$ are changing in vivo; measurements of total [ADP] might give an indication of the direction of change, but it is likely that more subtle but nevertheless important changes occur in the concentration of this metabolite in different compartments. Another emerging area is that of direct transfer of $Ca^{2+}$ between the SR and mitochondria in the heart, and it will be interesting to see whether this provides a major source of increases in $[Ca^{2+}]_m$ under physiological conditions; so far this is suggested from experiments using mitochondria isolated with attached SR components where $Ca^{2+}$ transfer from the SR-associated vesicle could increase NAD(P)H [64]. Finally studies over the last few years have suggested that stimulation of the citric acid cycle enzymes by increases in $[Ca^{2+}]_m$ during cardiac contraction may not be enough alone to provide the necessary increases in respiration to meet cardiac energy demands [76], and that ADP may after all be a major regulator of respiration via the localised concentration gradients where mitochondria and ATPases are organised into intracellular energy units [71,73]. However, there is as yet no method for detecting local changes in ADP. Without specific handles on the mitochondrial $Ca^{2+}$ transporters it remains impossible to unequivocally answer the question of whether they are essential in the regulation of respiration in the heart and other tissues.

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